THE CULTIVATION OF ONE GENERATION OF MALARIAL PARASITES (*PLASMODIUM FALCIPARUM*) IN VITRO, BY BASS'S METHOD

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PLATES XXIV, XXV

INTRODUCTION

From 1880, when Laveran discovered the malarial parasites, numerous workers have attempted their cultivation in vitro. It was not, however, until 1911 that Bass was able to state that he had succeeded in cultivating *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium falciparum*. In this preliminary paper the technique was not given in any detail, though certain factors were noted, such as incubation temperature of 40° C. or higher, strict anaerobic conditions and culture media of defibrinated or citrated human blood. Working on these lines, J. A. Sinton (1912), of the Liverpool School of Tropical Medicine, carried out a series of experiments, but in no instance was he successful in obtaining any development of the parasite, and it was only in October, 1912, that Bass's full technique became available.

After studying this paper, which appeared in the American Journal of Experimental Medicine for October, 1912, and after the examination of a series of eight slides kindly sent to the Liverpool School of Tropical Medicine by Dr. Bass, the results appeared so noteworthy that on the suggestion of Sir Ronald Ross we decided to make an immediate attempt to prove or disprove

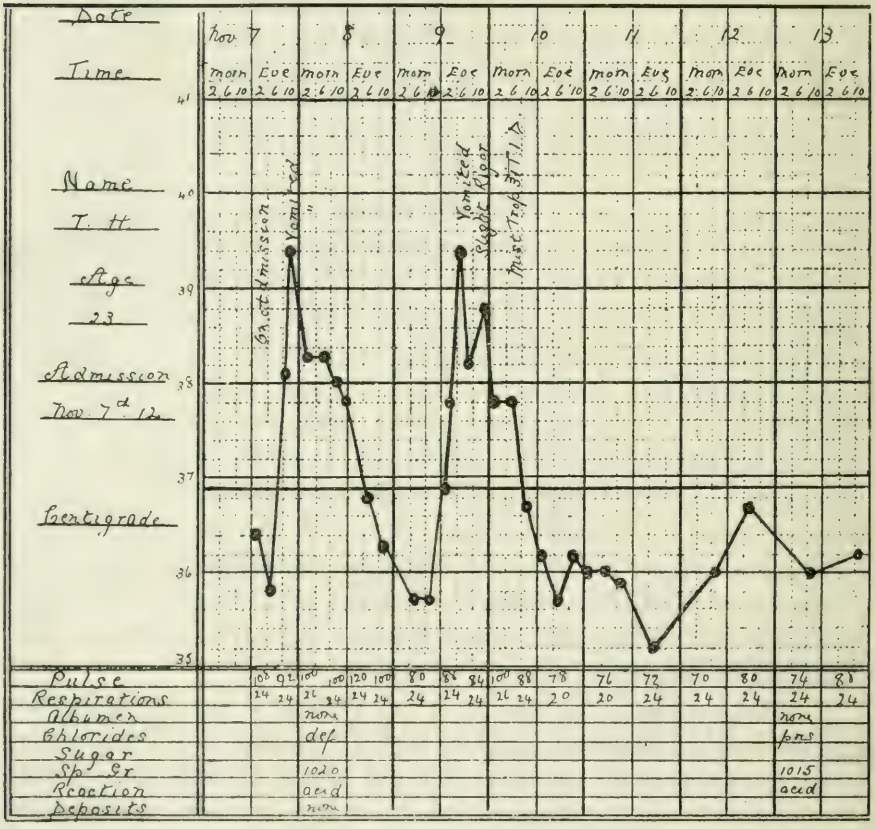
Bass's statements. We have much pleasure in thanking Sir E. Durning-Lawrence for generously supplying funds for this investigation.

With this end in view, therefore, we commenced our first set of experiments, and the results appear sufficiently remarkable to warrant this preliminary note.

CASES

The first case selected as suitable for attempting the cultivation of the parasite was a male, T. H., aet. 23, admitted to the Royal Southern Hospital in Sir Ronald Ross's clinic, November, 1912.

CHART I*



Mist. Trop. ℥i = 10 grains of quinine hydrobromide.

* Charts kindly prepared by Sister Marshall of the Tropical Ward, Royal Southern Hospital.

The following history was elicited. The patient had left the West Coast of Africa six weeks previously, having had a severe attack of malaria whilst at Sierra Leone. On the way home he had another attack and also suffered from gastric disorder and headache. Three weeks after his arrival in England, as he did not markedly improve in health and had several febrile attacks, he came to the Royal Southern Hospital, where his blood was examined, numerous small ring parasites being found. The temperature, which had been subnormal on admission, rose the same evening to 39·4° C., but next day again fell gradually to subnormal and remained so until 12 noon on the following day, when it again began to rise. On the blood being again examined, parasites were found to be numerous and 8 c.c. of blood were drawn off for cultivation from the median basilic vein. As the temperature rose the same night to 39·4° C., it will be seen that the blood was taken just at the onset of a second attack of fever. Ten grains of quinine hydrobromide were administered thrice daily, and the patient made a rapid and excellent recovery. See Chart I.

TECHNIQUE

Eight c.c. of blood were drawn from the median basilic vein, at the bend of the elbow, into a sterile glass syringe under aseptic precautions. In order to obtain and preserve this asepsis it is advisable that the arm be not washed with any watery solution, as two dangers have always to be guarded against, namely, that no micro-organisms (usually the *Staphylococcus albus*) be either carried into the vein or deposited in the culture tube, and, secondly, that no antiseptic shall be carried into the culture tube. We have always, therefore, found it advisable first to scrub thoroughly the arm with an alcoholic solution of biniodide of mercury, and secondly to wash off all the antiseptic and to fix the epidermal scales by a 70 per cent. solution of alcohol, afterwards allowing this to evaporate before puncturing. When the vein was punctured the blood was allowed to flow into the syringe, slowly forcing up the piston by means of its own pressure, after which it was transferred as quickly as possible (in order to prevent clotting) into a sterile culture tube. This tube was fitted with a thin glass rod running through the woollen plug and contained 1/10 c.c. of

a sterile 50 per cent. solution of Merck's dextrose. This dextrose is, according to Bass, a most important factor in the cultivation of the *Plasmodium*, and as it was not mentioned in his original paper, it is probable that Sinton's failure was due to its omission.

The blood is next gently defibrinated and at the same time mixed with the dextrose by gently stirring with the glass rod, care being taken to avoid air bubbles in any of the manipulations.

We next transferred it to two culture tubes, the column of blood being one to two inches thick, and incubated at 38° C. The red blood cells gradually settled and about half an inch of serum accumulated on the surface.

The above technique is practically identical with that described by Bass, the only points of difference to be noted being:—

(*a*) The slight increase in the amount of dextrose employed relative to the quantity of blood—(1/10 c.c. dextrose to 10 c.c. blood is recommended by Bass).

(*b*) The lower temperature at which we incubated (40° C. advised by Bass).

The tubes so prepared contain roughly three layers, namely:—

(1) A top layer of varying depth, preferably about half to one inch of clear serum.

(2) A thin intermediate layer of loose red corpuscles and white cells—the culture layer.

(3) A bottom layer consisting mainly of red cells, in which the parasites die in periods varying from two to twenty hours.

EXAMINATION OF CULTURES

By means of a drawn-out capillary pipette, a few of the surface cells from the intermediate layer in our cultures were drawn off at twelve hours, twenty-five hours, twenty-seven hours and thirty-two hours. Small drops were placed on clean glass slides and smears made, fixed in methyl alcohol for twenty minutes, and stained in the usual way with Giemsa or modified Romanowsky solution. Controls were made in each case.

It was found that the smears stained well, but it is advisable to make somewhat thick smears, owing to the amount of serum mixed with the cells.

MORPHOLOGY

(A) *Control.* The blood examined before incubation showed numerous typical small ring parasites with nucleus and vacuole. The character of the nucleus varied in shape, being either circular or rod-shaped, and was situated in the vacuole, as a rule near one margin. The parasites varied in size with a maximum diameter of about 3μ. They also varied in shape, though the ring form predominated. Amoeboid movement was seen in some of the irregular forms, but pigment, segmenting forms and crescents were all absent. There was no enlargement of the red blood corpuscles and no stippling of the red cells.

The amoebae were typical of the malignant tertian parasite, *Plasmodium falciparum* (Pl. XXIV, figs. 1-5).

It is to be noted, in regard to the description of the forms cultivated by us in vitro, that the malignant tertian parasite is seldom or never seen undergoing schizogony in the peripheral circulation; as a rule, only small rings are present in this situation, segmentation occurring almost entirely in the internal organs. The active small rings grow larger in size and become the less amoeboid trophozoite, finally reaching the stage of the mature schizont with a single nucleus. The nucleus then divides up by a primitive form of mitosis, and, as the nuclei increase in number, division becomes simpler (Schaudinn). Immediately previous to the division of the nucleus the presence of pigment makes itself manifest. It is round this central mass of pigment that the merozoites formed by the nuclear division are arranged, until the final stage of the fully segmented parasite, when the enclosing corpuscle shews signs of breaking down.

By the cultivation of the parasite in vitro we have been enabled to observe the process of segmentation actually occurring in the culture tubes, and thus the old, difficult, and mostly inaccurate method of studying it in the internal organs has been avoided.

(B) *Culture,* twelve hours duration; 38° C.; fixed specimens examined. A definite increase in the size of the parasite was found to have occurred, the measurements varying from 5μ to 6·5μ. A round mass of pigment about 1μ in diameter was present in almost every case—usually at one pole of the parasite. The chromatin

had also increased, in some cases retaining its original compact form, in others spread out irregularly (see figs. 6-13). No distinct amoebae could now be seen. At this stage of the cultures an interesting and well-marked feature was the tendency which the corpuscles containing parasites shewed to clump together into irregular masses (see Pl. XXIV, fig. 10, and Pl. XXV, figs. 2 and 3). On one slide a mass of as many as sixty parasites was made out. There was no marked increase in the size of the containing corpuscles.

The shape of the organisms varies, being circular, irregular and (most frequently) oval. One young crescent was found (fig. 14). This, however, may have been originally in the peripheral blood, as we have noted in other cultures that crescents persist for many hours without shewing any apparent change.

(C) *Culture*, twenty-five hours incubation, 38° C.

A most remarkable appearance was now noted in fixed specimens prepared from two culture tubes, definite segmenting forms being seen in large numbers, shewing the nucleus broken up into fragments varying from two to thirty in number. A microphotograph is herewith reproduced shewing a portion of a slide on which hundreds of these schizonts were seen clumped together (Pl. XXV, figs. 4 and 5). The diameter of these forms varied according to the amount of segmentation that had taken place, the maximum being about 7 to 8μ. In many the corpuscle could not be seen, but in others the margin of the enveloping red cell could be distinctly seen (Pl. XXIV, figs. 15-30, and Pl. XXV, figs. 4, 5). The pigment mass is quite distinct, and where segmentation is nearly complete the merozoites are arranged concentrically round it, thus forming the so-called 'rosette.' Fig. 23 shews the formation of thirty merozoites.

No evidence of these merozoites having entered new corpuscles, and so beginning another generation of parasites, was obtained.

(D) *Culture*, twenty-seven hours incubation.

Only a few segmenting forms were now found, the probability being that the corpuscles had burst and the merozoites dispersed, thus allowing the destructive action of the serum and leucocytes to

take place. Of the few found, fig. 32 shews a parasite with early segmentation, and fig. 31 the nucleus broken into sixteen daughter portions.

(E) *Culture*, sixty-six hours incubation.

It now became increasingly difficult to find parasites, but one is shewn with the nucleus divided into four (fig. 30). Slides examined after this time shewed only disintegrated and degenerate forms, all the parasites having apparently perished.

A second series of experiments was carried out on the blood of a patient, W.B., from the West Coast of Africa, who gave a history of six weeks' illness. He habitually took five grains of quinine per diem, and after landing at Liverpool came direct to the Royal Southern Hospital.

On admission his temperature was 38·7° C., and he seemed very ill with definite anaemia. The blood examination shewed a few small ring parasites (*Plasmodium falciparum*). No quinine was given, and the temperature fell gradually to subnormal on the following day and then began to rise. 10 c.c. of blood was drawn off, as in the previous case, just as the temperature was rising, and cultures prepared as already described. (See Chart II.)

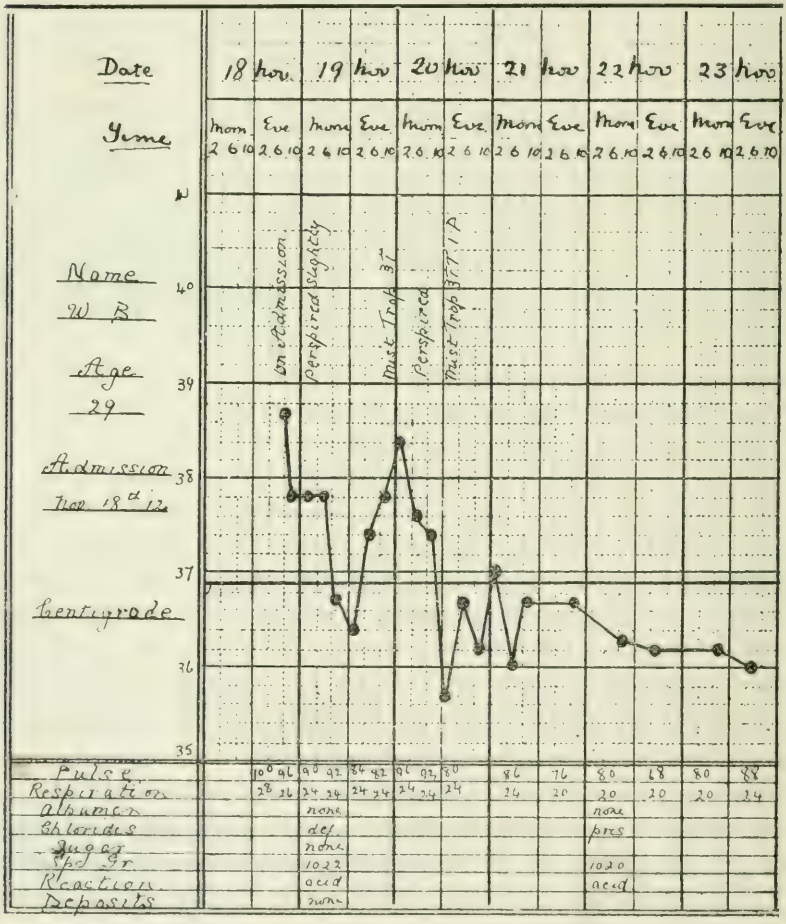
(A) *Control.* Before incubation.

A few small ringed parasites were found, but much less numerous than in the previous case.

(B) *Cultures.* After seven hours incubation, temp. 39° C., the parasites were distinctly enlarged. In eighteen hours many early segmentation forms were found with the nuclei broken up into two or four. Segmentation proceeded steadily, slides being examined after twenty-two, twenty-five, thirty and thirty-two hours, after which development apparently ceased. In some of these later slides small parasites were seen without a vacuole, but with distinct nucleus and protoplasm, suggesting the possibility that several of the merozoites had escaped destruction and had entered a fresh corpuscle. It is impossible, however, to be dogmatic about this, as no definite increase in the number of small rings was noted in any of our slides after maximum segmentation had occurred.

In this second series of culture tubes the segmentation did not appear to proceed so rapidly as in those of the first case, nor did the nucleus break up into as many segments. The schizonts also appeared smaller in diameter and did not fill the corpuscles so completely. In this connection it is interesting to note that the patient, up to the time of admission, had been taking quinine with great regularity. This fact leads us to suggest that possibly the vitality of the parasites had been impaired by the drug, sufficiently at any rate to prevent full segmentation and to account for the difference in growth noted between the two cases.

CHART II



Mist. Trop. ℥i = 15 grains of quinine hydrobromide.

The patient made a good recovery on quinine being pushed, and is almost well at the time of writing.

REMARKS

Such is the morphology of our two cases of *Plasmodium falciparum* infection, cultivated in vitro. If our observations are correct, it appears to afford a simple method of studying some of the hitherto difficult problems of malaria. For example, the segmentation and sporulation of *P. falciparum* may be thus studied. It will be remembered that Sir Ronald Ross (1910) in his book 'The Prevention of Malaria,' states that 'the number of spores produced by each species of parasite is variable,' and that 'different authors give different figures, and in all probability the number has never been accurately estimated.' Sir Ronald adopts the figures of Welch (1897), who says that the 'malignant parasite produces six to twenty or more spores every two days.' In the work of Marchiafava, Bignami and Mannaberg (1894) the following description of the segmentation of the aestivo-autumnal parasite is given. 'The forms which precede fission (*i.e.*, segmentation) are represented by round or ovoid parasites which are in size between a quarter and half that of the red blood corpuscle, having pigment collected at the centre or slightly excentric in a small mass or in a cluster of granules in motion. The forms of fission vary in size; they may be as large as two-thirds of the red blood corpuscle, and are composed of one or two circles of spores (usually ten or twelve, seldom fifteen to sixteen) arranged round the central mass of pigment.'

Stephens and Christophers, in 'The Practical Study of Malaria' (1908), say that the malignant tertian parasite is rarely seen segmenting in the peripheral circulation, and state that there are eight to ten chromatin masses.

It is thus apparent that different observers have, in their studies of segmentation, encountered parasites with varying numbers of chromatin masses. This diversity of number is capable of various explanations:—

(1) That there are two or three varieties of the malignant parasites.

(2) At the time the observations were made segmentation may have been more or less complete.

(3) The administration of quinine may have in some way lowered or modified the vitality of the parasite, and thus prevented complete segmentation.

It is obviously impossible to dogmatize on such a limited number of cases and in such a short paper. We are still proceeding with our experiments with a view to elucidating these and several other important points which are not explained in Bass's paper. Thus:

(1) Why should the parasite only grow on what we have called the thin intermediate layer in the culture tubes, that is, the layer immediately beneath the serum and above the main mass of red blood cells; in other words, the layer in most immediate contact with the potentially destructive leucocytes and serum?

(2) What is the function of the apparently inert mass of red cells immediately beneath the growing layer, and of the serum immediately above? In both the cases we mention, one would expect the serum to be filled with antibodies, produced by repeated sporulations of parasites, and yet it is apparently powerless to stop in any way parasitic growth as long as such growth remains intra-corpuscular.

(3) Do the parasites grow better or worse in normal human serum? In other words, is there any specific affinity between the vitality of the individual's red cells and his own serum?

Such, and many other questions, can only be settled by repeated experiment. We have ventured to bring forward these two consecutive cases of the growth of a single generation of one particular variety of malarial parasite mainly because they show many interesting morphological points, which so far have not been touched on by Bass in his published papers. Also, they appear to prove to us, and to various competent observers to whom the slides have been shown, that the growth of the malarial parasite *in vitro* has at last been successfully accomplished.

We wish to thank Dr. H. B. Fantham for his valuable advice and help during this investigation.

NOTE

I have examined both the specimens sent to me by Dr. Bass from Tulane University, U.S.A., and those made by Drs. Thomson and McLellan in Liverpool, and am quite convinced that they give indisputable evidence of the successful cultivation of *Plasmodium falciparum* up to the sporulating forms. These forms are much too numerous in the specimens to admit of the supposition that the parasites have not developed since the blood was taken from the patient. The advance thus made is one of great importance, as all efforts to cultivate the parasites of malaria have hitherto failed since their discovery by Laveran in 1880.

RONALD ROSS.

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EXPLANATION OF PLATE XXIV

Figures drawn by means of an Abbé camera lucida, using Leitz ocular 4 and objective 1/12 inch. Stained with Giemsa or Romanowsky. Magnification 1,600 diameters.

Case T. H.

Figs. 1-5.—Parasites from the peripheral blood before incubation. Stained with Romanowsky. Fig. 5 shows elongated form.

Figs. 6-13.—Parasites after twelve hours cultivation. These show definite increase in size. The chromatin is more spread out, and a distinct circular mass of pigment is to be seen. Fig. 10 shows four corpuscles clumped together, each containing a parasite. (Romanowsky stain.)

Fig. 14.—A young crescent stained with Giemsa. This was found after twelve hours incubation, and was probably present in the original blood.

Fig. 15.—A schizont with the chromatin divided into two. After twenty-five hours incubation (Giemsa stain).

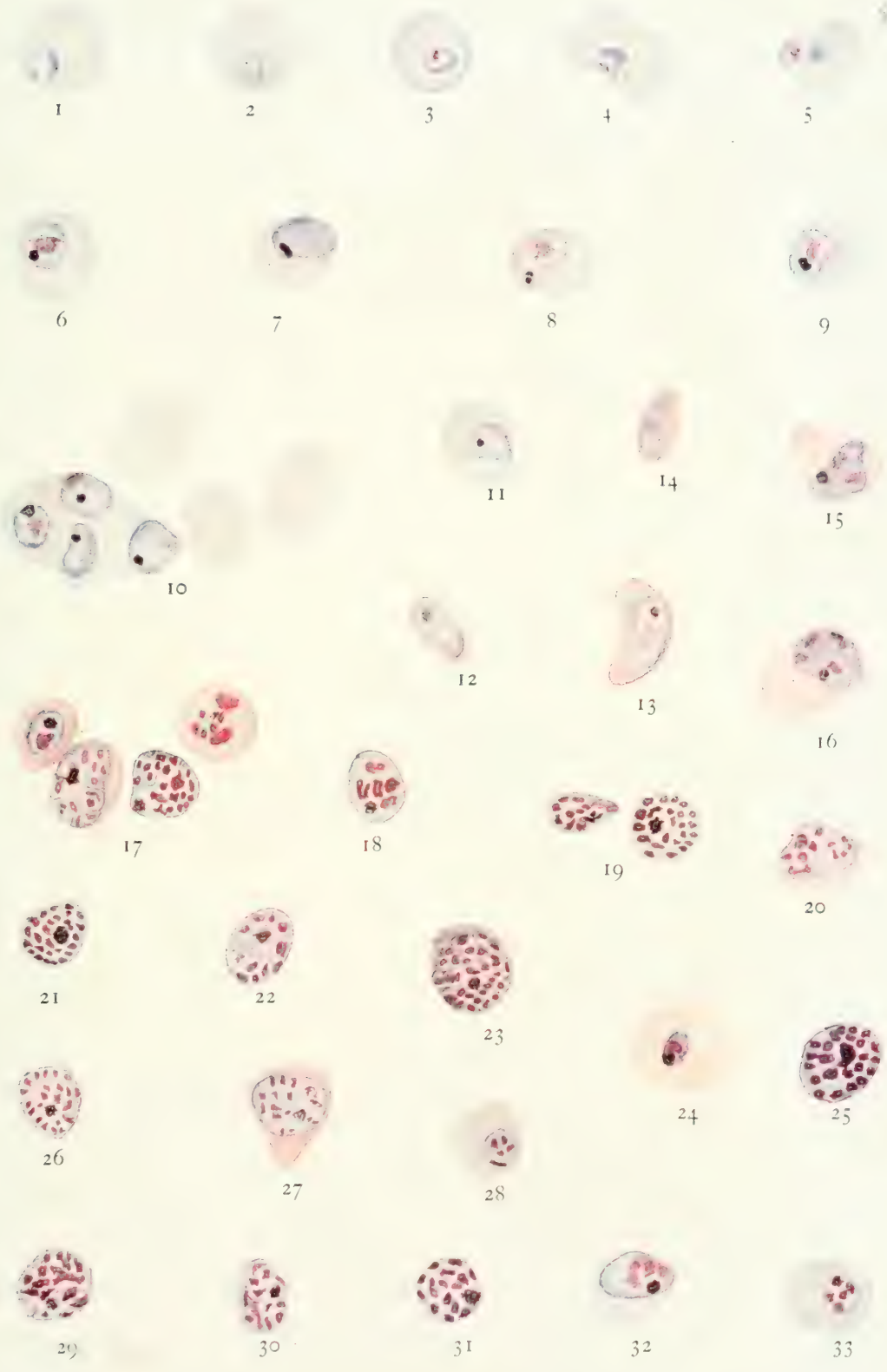
Fig. 16.—A schizont with the chromatin divided into four. After twenty-five hours incubation (Giemsa stain).

Figs. 17-30.—Various stages of segmentation of nucleus—all found in a twenty-five hours culture. Fig. 23 shows thirty chromatin particles.

Fig. 31.—A segmenting form found after twenty-seven hours culture.

Fig. 32.—Form after twenty-seven hours culture.

Fig. 33.—Form after sixty-six hours in culture.



CULTIVATION OF *PLASMODIUM FALCIPARUM*

J. G. Thomson, del.

C. Tinling & Co., Ltd., Liverpool.